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Worm peptidomics

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ABSTRACT

Bioactive peptides are present in all metazoan species where they orchestrate diverse functions. In the last decade, high-throughput approaches based on mass spectrometry helped the identification of endogenously occurring peptides in different species. We here review biochemical strategies to obtain sequence information of natural (non-tryptic) peptides in *Caenorhabditis elegans* and in the related nematodes *Caenorhabditis briggsae* and *Ascaris suum* with particular attention for sample preparation and methodology. In addition, we describe seven new *C. elegans* neuropeptides that we recently discovered by sequencing additional peptides. Finally, we explain how differential peptidomics approaches were used to characterize key neuropeptide processing enzymes.

1. Introduction

The nematode *Caenorhabditis elegans* is a common model system in neuroscience research for several reasons. It was the first multicellular organism from which the genome was entirely sequenced. Comparison of *C. elegans* genes with vertebrate homologues showed many similarities [1,2]. In fact, essential neuronal processes and functions (including neurotransmission at chemical synapses, electrical signalling through gap junctions and extrasynaptic communications *via* neuropeptides) are highly conserved among all animals. The hermaphrodite nematode has exactly 302 neurons of which the cell lineage and exact physical locations are known. Reconstructions of electron micrographs allowed mapping all synaptic connections and gap junctions and as such, the nervous system can be considered as "hard wired" [3]. However, predicting behavioural output solely based on the wiring diagram is not straightforward, especially as synapses can be excitatory or inhibitory. In addition, slow-acting transmitters such as neuropeptides should be considered as a third layer of information flow in neuronal communication, next to chemical synapses and gap junctions. In this respect, it is very likely that nearly all neurons secrete neuropeptides and many roles for peptides in key physiological processes and behaviours have been characterized [4–6].

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We here review the efforts that have been undertaken to reveal the rich diversity of neuropeptide-encoding genes, neuropeptide processing enzymes, the resulting bioactive peptide sequences and characterization of GPCRs that ultimately aided further functional elucidation of selected neuropeptidergic signalling systems in *C. elegans*.

2. Three families of neuropeptide-encoding genes

2.1. *FMRFamide-like peptides (FLP)*

In *C. elegans*, three families of neuropeptides or neuropeptide-encoding genes are generally considered. Bioactive peptides displaying carboxyterminal sequence similarity with the cardioexcitatory peptide FMRFamide, which was first discovered in a bivalve mollusk [7], belong to the family of FMRFamide-like peptides (FLP), also referred to as FMRFamide-related peptides (FaRP). This diverse family is widely represented in various metazoan species [8], including insects [9], nematodes [10,11] and platyhelminths [12]. Whole mount immunocytochemistry using antibodies that recognize the carboxyterminal signature of FaRPs allowed visualization of immunoreactive neurons in *C. elegans*, hereby suggesting the presence of FaRPs in the nematode [13–16]. Indeed, bioinformatics surveys of cDNA libraries and BLAST allowed uncovering nearly 30 *flp* genes in *C. elegans*. *flp-1* to *flp-23* and *flp-28* were found by the group of Chris Li [6,16–19], while the lab of Aaron Maule reported the identification of *flp-24* to *flp-27* and *flp-32* [10,11]. In addition, peptides from *flp-24*, *flp-26*, *flp-33* and *flp-34* could be detected by LC–MS-based peptidomics approaches in the Schoofs group [20,21] (see further).

2.2. *Insulins (INS)*

A second family comprises 40 peptides (INS-1 to INS-39 and DAF-28) with sequence resemblance with insulin [22–25]. Expression of *ins-1* through *ins-31* and *ins-33* have been confirmed on the level of cDNA sequences and reporter constructs revealed cellular localization patterns for 15 *ins* genes [25,26]. Nevertheless, no single *C. elegans* INS peptide has ever been biochemically characterized.

2.3. *Neuropeptide-like proteins (NLP)*

The so-called neuropeptide-like protein (*nlp*) genes were initially identified in *C. elegans* by the group of Anne Hart [27] and in other nematodes by the Maule lab [28] based on structural criteria and sequence resemblance with peptides (*e.g.* allatostatin, myomodulin, buccalin, orcokinin) from other species. This way, 32 *nlp* genes were identified and expression patterns were examined using GFP reporter constructs. Resulting peptides can be further subdivided based on sequence motifs like FAFA, GGXY-amide, MRX-amide, LQF-amide, LXDX-amide and GGARAF. Interestingly, some *nlp* genes appear to be expressed in the epidermis and especially *nlp-29*, *nlp-31* and *nlp-33* were differentially regulated in the event of a fungal and/or bacterial infection. They were therefore considered as antimicrobial peptides (AMP), as are the structurally related caenacins (CNC) [29,30]. Obviously, AMPs, including some NLPs, CNCs, ABF peptides, fungus-induced peptides (FIP) and FIP-related (FIPR) peptides should be considered as naturally occurring bioactive peptides, but are discussed elsewhere [31]. Later-on, many additional NLP peptide-encoding genes (until *nlp-52*) could be identified by further mining the *C. elegans* genome and by using LC–MS based peptidomics approaches [20,28,32–34] (see below).

3. Biochemical characterization of FLP and NLP peptides

3.1. *Classical biochemical approaches*

As explained before, a lot of efforts mining EST libraries or genomic data allowed identifications of numerous neuropeptide-encoding genes. However, it is still hard to predict exact sequences of the encoded bioactive peptides. Traditionally and before the genomics era, bioactive peptides were typically identified and characterized by screening of HPLC fractions using a dedicated (bio)assay. After several rounds of chromatographic separations, the active compound could be purified and sequence identity was revealed by Edman degradation. In doing so, 12 FLP peptides could be identified by radioimmunological screening of peptide extracts from *C. elegans* [35–40]. Using similar approaches, 20 FLP peptides could be isolated from the larger nematode *Ascaris suum* where they are annotated as AF peptides [41–44].

3.2. *C. elegans peptidomics using 2D-nanoLC Q-TOF MS/MS*

To reveal peptide sequences in a high-throughput manner, a peptidomics approach combining two-dimensional nanoscale liquid chromatography (2D-nanoLC) and quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) was performed in the Schoofs lab. This setup, as depicted in Fig. 1, was first optimized when exploring the neuropeptidome of the fruitfly *D. melanogaster* [45,46]. A detailed protocol to extract naturally occurring peptides from *C. elegans* can be found in [47]. In short, worms were placed in an acidified methanolic extraction solvent (methanol/water/acetic acid 90/9/1), homogenized and sonicated prior to centrifugation. The pellet was discarded and methanol was evaporated using a Speed Vac concentrator. Next, the aqueous residue was delipidated by liquid–liquid extraction with an equal volume of ethyl acetate or n-hexane. Finally, the peptide extract was desalted by solid phase extraction.

In total, 60 peptides could be identified, including peptides missed by the initial bioinformatics surveys [20]. Though accurate sequences of many of the previously predicted peptides could be confirmed, shorter or longer forms could also be observed. For example, AMDSPLIRFamide from the FLP-13 precursor was found, though the longer form, SDRPTRAMDSPLIRFamide, was initially predicted. Probably the arginine residue at position six acts as a monobasic cleavage site to be recognized by proprotein convertases (see below). Revisiting these 2D-nanoLC MS/MS data allowed characterizing two additional peptides that were initially overlooked.

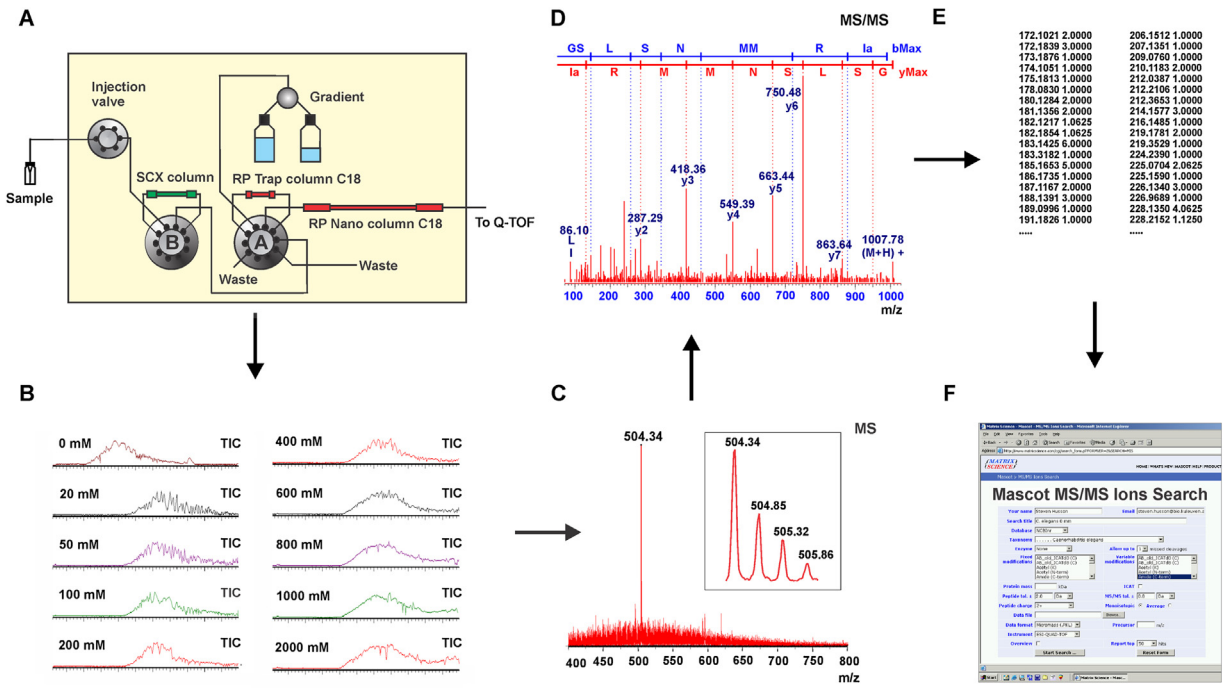

Fig. 1 – 2D nanoLC Q-TOF MS/MS workflow for *C. elegans* peptidomics. Initial *C. elegans* peptidomics experiments were performed using an Ultimate HPLC pump, a Switchos column-switching device and a Famos autosampler (all LC Packings). This LC-setup (A) was coupled to a Q-TOF hybrid Quadrupole time-of-flight mass spectrometer (Micromass), as described in [20]; total ion currents (TIC) are shown in (B). Peptides that were first loaded on a strong cation exchange (SCX) column (Bio-SCX, 500 μm × 15 mm, LC-Packings) could be eluted by 10 salt plugs ranging from 0 to 2000 mM of ammonium acetate. Next, each fraction underwent a second separation on a nanoscale C18 column (3 μm, 100 μm × 100 mm, Atlantis, Waters) using a 50 min gradient to 50% ACN containing 0.1% FA at a flow rate of 200 nl/min. The column eluent was directly connected to the electrospray (ESI) source of the Q-TOF instrument that automatically selected doubly and triply charged parent ions when intensities increased above a predefined threshold for fragmentation aided by argon gas (C and D). MS/MS data were transformed into peak list files (E) to be submitted to Mascot as search engine (F).

GVGDVPSMFFSPFRMMamide originates from the C38C10.6 precursor that we propose to name NLP-48. This peptide is flanked by a RX_2R at the aminoterminus and by (G)KR at the carboxyterminus. SPSMGLSLAEYMASPQGGDNFHFMPSamide is contained in the H05L03.3 gene product which we propose to name NLP-49 and which is, respectively, flanked by KR and (G)RK at amino- and carboxytermini. Both new neuropeptide precursors contain a signal peptide recognized by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP-4.0) (Fig. 2A).

3.3. *Identification of five novel peptides in C. elegans by off-line HPLC–MALDI TOF/TOF MS*

Some peptides ionize more efficient by matrix-assisted laser desorption ionization (MALDI) compared to ESI, and *vica versa*. Hence, additional peptides can be identified by employing another peptidomics workflow where HPLC fractions are collected and analyzed by MALDI-TOF MS/MS. (Ultraflex II, Bruker Daltonics). This simple, though robust approach was also used to explore the peptidome of the related nematode *Caenorhabditis briggsae* [48] and to compare peptide profiles from different *C. elegans* strains with defective neuropeptide processing enzymes [21,49–51], as explained later-on. Ion peaks were initially annotated by comparing the masses of the ions detected with the theoretical masses of *in silico* predicted (and previously identified) neuropeptides. To obtain sequence information, selected ions were fragmented and resulting peak list files were submitted to a database search (using mascot) against the protein database of *C. elegans* (Matrix Science). Doing so, we here report the identification of five novel (neuro)peptides: TEGLSRASANAYYRLamide originates from the F41G3.1 precursor that we would like to annotate as NLP-50; SQTQEANIQPFIRF (note that no amide is present at the carboxyterminus) originates from T24F1.5 that we would like to annotate as NLP-51; GDVKSVFFSPFRM-Vamide is contained in the F42G9.4 precursor that we propose to name NLP-52. Finally, ADISTFASAINNAGRLRYamide and ALNRDSLVASLNNAERLRFamide are contained in the R09A1.5 precursor that we would like to name FLP-34, as the last peptide contains the typical RFamide motif at the carboxyterminus. All new neuropeptide precursors contain a signal peptide as verified by SignalP 4.0 (Fig. 2A and B–F shows fragmentation spectra).

3.4. *Peptidomics of the related nematode C. briggsae by off-line HPLC–MALDI TOF MS*

C. briggsae is the closest studied species of nematode to *C. elegans* and shares the hermaphroditic mode of reproduction. They diverged about 100 million years ago and are

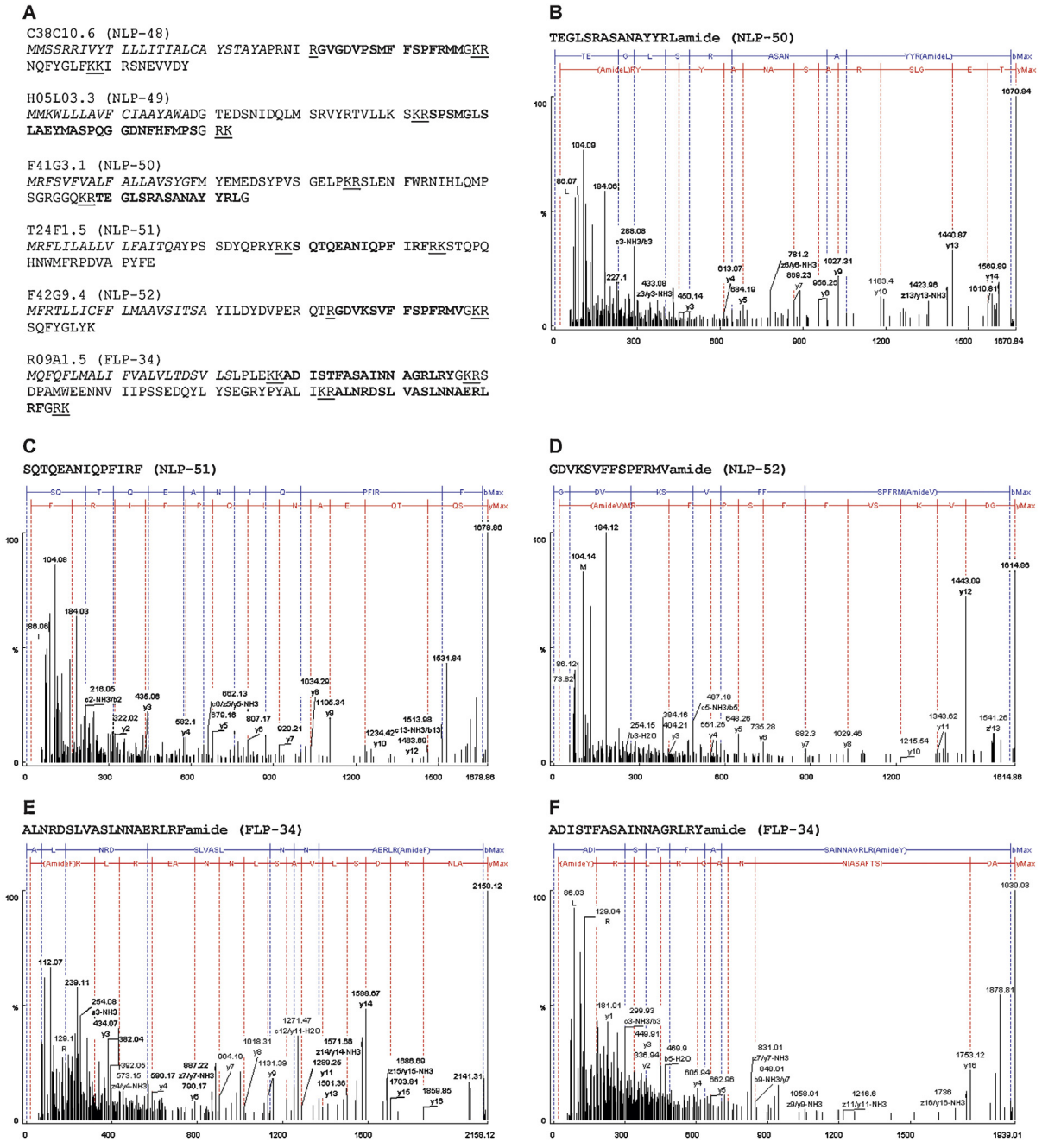

Fig. 2 – New *C. elegans* neuropeptides. We here report the identification of 7 new neuropeptides that originate from 5 NLP precursors and 1 FLP precursor, as displayed in (A). The signal sequence is shown in italics, dibasic cleavage motifs are underlined and the identified peptide is in bold. Peptides from NLP-48 and NLP-49 could be identified by resubmitting previously generated 2D nanoLC MS/MS data [20] to an updated *C. elegans* protein database. Additional peptides could be identified by off-line HPLC MALDI-TOF/TOF MS; see (B-F) for resulting fragmentation spectra. Extracts were separated on a Symmetry C18 column (2.1 mm × 150 mm, 3.5 μm at a flow rate of 300 μL/min) using a linear gradient from 2 to ~50% ACN containing 0.1% TFA in 60 min to allow collecting 60 fractions. About ½ of the fractions were dried in a Speed Vac concentrator and resuspended in 1 μL MQ/ACN/TFA (50/49.9/0.1) to be spotted on the stainless steel MALDI target plate and mixed with 1 μL of matrix consisting of a saturated solution of α-cyano-4-hydroxy cinnamic acid in acetone. Spectra were recorded in MS mode using an UltraflexII instrument (Bruker Daltonics), within a mass range from m/z 500 to m/z 3000 or 4000. For initial annotation of ion peaks, we used a custom-made software programme, VglP, which automatically compares the generated peak list files with the theoretical masses of *in silico* predicted (and previously identified) neuropeptides. In order to obtain sequence information, selected ion peaks were fragmented using an optimized LIFT (TOF/TOF) procedure. Next, all MS/MS spectra were automatically processed (background subtraction, smoothing and peak picking; by using FlexAnalysis, Bruker Daltonics) to generate peak list files to be submitted to a "Mascot" search against the protein database of *C. elegans* (matrix science).

nearly morphologically identical (for an un-experienced eye). Surprisingly, however, its genomic sequence revealed many differences [52–55]. As 5 years ago no neuropeptide genes were annotated in the *C. briggsae* genome, its peptidome was explored by combining *in silico* analysis with LC–MS to identify 27 *flp* and 31 *nlp* genes [48]. Off-line screening of HPLC fractions by MALDI-TOF MS (as explained above) revealed the presence of 37 FLP peptides and 22 NLP peptides. In addition, this peptidomics approach allowed obtaining better insights on splicing of (sometimes wrongly) predicted exons. For example, the peptide SPAQWQRANGLWamide containing a typical $W(X)_6$ Wamide motif that is also present in the myoinhibiting peptides (MIPs) from insects, was initially found in *C. elegans* by LC–MS/MS [20] and is encoded by the last exon of *nlp-38*. Although this peptide did not appear to be present in the predicted *C. briggsae* homologue, the same MIP-like peptide could be sequenced from a peptide extract of *C. briggsae*. As the corresponding DNA sequence could be (manually) found in the same nucleotide sequence contig, it is likely that the last exon of the *C. briggsae* neuropeptide precursor gene was not annotated as such due to wrong *in silico* splicing.

3.5. *Direct peptide profiling of A. suum tissues by MALDI-TOF, -TOF/TOF or ICR-FT MS*

The parasitic nematode *A. suum* contains 298 neurons and is significantly larger than *C. elegans*, facilitating physiological characterizations of neurons and muscles. As already mentioned above, 20 endogenous peptides could be biochemically characterized from this large roundworm by classical purification strategies and monitoring reactivity with a polyclonal antibody against the FMRFamide motif [41–43]. About ten years ago, three additional AF peptides were sequenced directly from the tissue by MALDI-TOF MS [56]. Direct peptide profiling of dissected neuronal *Ascaris* tissues allowed spatial mapping of known and predicted peptides without the need for extensive extraction procedures [56,57]. In 2005, the Stretton group was able to identify 41 peptides originating from the nerve ring (NR), ventral ganglion (VG), retrovesicular ganglia (RVG), dorsal ganglion (DG), lateral line ganglia (LLG) the ventral and dorsal nerve cords (VC, DC) and ventrodorsal commissures (VDCs) by employing a MALDI-TOF (Reflex II, Bruker Daltonics), MALDI-TOF/TOF (UltraflexII, Bruker Daltonics) or a MALDI-FT (Ion Spec Fourier Transform MS, equipped with a 7 T magnet and ability for sustained off-resonance irradiation and collision-induced dissociation (SORI-CID)). Assignments of peptide sequences were made when the observed *m/z* value was within 0.5 Da; 0.2 Da or 0.001 Da (as measured on the Reflex II, Ultraflex or FT instrument, respectively) of the theoretical values of the peptides. In the event that the mass of a peptide is not a unique identifier to unambiguously assign a peptide sequence, additional confidence was gained from chemical derivatizations (*i.e.* acetylation of primary amine groups and oxidation of methionines) performed on the tissues prior to MS analysis. Peptide profiles from different neuronal structures showed significant overlap in the peptide content, though distinct peptides could be observed for each tissue. In general, the NR, VG and RVG exhibited the largest spectra complexities and intensities, in contrast to the low signal-to-noise ratios in spectra obtained from the VDCs, which is likely due to low peptide abundances. Five years later, the Stretton group was able to map neuropeptide expression in single dissected neurons (ALA and RID) from the DG [58], where the availability of genome survey sequences (GSSs) have been extremely helpful in aiding *de novo* sequencing. Similarly, several peptides could be measured in AVK neurons by MS and their presence could be confirmed by *in situ* hybridization and immunocytochemistry [59].

3.6. *Peptidomics of A. suum by data mining and nanoLC–LTQ Orbitrap MS/MS*

After publication of the draft genome sequence of *Ascaris* [60], the Stretton group combined an *in silico* data mining approach with a high-throughput peptidomics experiment to report 21 novel peptides, in addition to many previously identified peptides [61]. Heads were freeze-powdered and peptides were extracted using the "typical" 90/9/1-extraction solvent (methanol/water/acetic acid) as indicated above, followed by delipidation by liquid–liquid extraction with 15/85 ethyl acetate/hexane and solid phase extraction using C18 cartridges. Finally, size exclusion separations were performed to remove proteins. For LC–MS analysis, the nanoLC system (nanoAcquity, Waters) was connected to an ESI FT/ion-trap MS instrument (LTQ Orbitrap Velos, Thermo Fisher Scientific) and MS/MS data from collision induced dissociation (CID), high energy collision dissociation (HCD) and electron transfer dissociation (ETD) were searched against an in-house peptide database using SEQUEST or Mascot. Overall, sequences of previously identified or predicted peptides could be confirmed, though shorter forms or incompletely processed variants were also found, similar to previous results obtained from *C. elegans* [20]. For example, the *A. suum* peptide ENEKKAVPGVLRFamide results from an incomplete cleavage of AF3. In contrast, LM(oxidized)DPLIRFamide is a shorter form or fragment of AF34 (DSKLMDPLIRFamide), which is encoded by *afp-13*. The internal lysine at position three of AF34 thus likely acts as a monobasic cleavage site to be recognized by proprotein convertases or other enzymes. Alternatively, the full-length peptide might have been subject to *in vivo* or *in vitro* degradation, hereby yielding a truncated peptide.

4. Characterization of neuropeptide processing enzymes

4.1. *Biosynthesis of bioactive peptides: insights from peptidomics experiments in other organisms*

As exemplified above, mining the genome for neuropeptide-encoding genes and *in silico* prediction of accurate peptide sequences is not straightforward, especially as biosynthesis of bioactive peptides requires multiple post-translational processing steps. By characterizing genes required for neuropeptide biosynthesis, specific roles can be identified for neuropeptides in the generation of behaviour *in vivo*. In addition, processing enzymes are considered to be attractive targets for the development of powerful novel therapeutics [62]. The formation of bioactive peptides is a multistep process that starts with the synthesis of larger inactive preproproteins

or peptide precursors in the endoplasmic reticulum. First, the aminoterminal signal peptide is chopped off from the inactive proprotein precursor protein after entry into the secretory pathway. Next, proprotein convertases (PC) recognize typical cleavage motifs consisting of basic residues like arginine and lysine. KR and RR are mainly observed, while RK, KK or (R/K)$X_{2/4/6/8}$(R/K) are found in lower frequency [63]. In mammals, 7 PCs (furin, PC1/3, PC2, PC4, PC5/6, PC7/8/LPC and PACE4) have been described [64–66]; all of which show sequence similarity to the yeast prototype Kex2p [67]. PC2 and PC1/3 recognize substrates with dibasic residues and are thus key PCs needed for the processing of neuropeptide precursors [64–66]. Indeed, peptidomics analysis of PC1/3 [68] and PC2 [69] knockout mice revealed clear reductions of neuropeptides present. Comparison and analysis of cleavage motifs revealed that while PC1/3 and PC2 have redundant substrate preferences; preferences for each PC can be distinguished by particular site residues. In the fruitfly, three subtilisin-like enzymes appear to be present, of which *amontillado (amon)* encodes for the *Drosophila* PC2 homolog; a PC1/3 homolog seems to be lacking [70]. By semi-quantitative MALDI-TOF mass spectrometric profiling in combination with genetics and immunostainings, AMON was proven to be the major neuropeptide processing proprotein convertase in the CNS of the fruitfly [71]. In addition, all entero-endocrine peptides from the *Drosophila* midgut seemed to be processed by AMON as well, as respective mutants display a strongly impaired peptide profile [72].

In general, PC2 requires proteolytic activation by the aminoterminal part of the chaperone protein 7B2, while the carboxyterminal fragment of 7B2 functions to inhibit the activity of PC2 [73,74]. Interestingly, deficiency of 7B2 does not mimic phenotypes observed in PC2 knock-out mice models. 7B2-null mice die early from Cushing's disease in addition to many other atypical symptoms [75], while PC2-null mice, though having a different genetic background [76], are viable [77]. In line with these phenotypically differences, striking differences in peptide profiles could be observed in PC2-null and 7B2-null mice [75,77]. *Drosophila* PC2 (AMON) also requires 7B2 for its activation [78], though further evidence from peptidomics experiments is lacking. Recently, 7B2 was also shown to suppress formation of amyloid-β (Aβ)-derived peptides and α-synuclein aggregation, which are characteristic for the neurodegenerative diseases Alzheimer and Parkinson, respectively [79].

After cleavage of the proprotein peptide precursor at basic recognition motifs by PCs, the resulting intermediate peptides are further processed by a carboxypeptidase that chops off the carboxyterminal basic amino acids. Mammalian carboxypeptidase E (CPE) was initially discovered in 1982 as enkephalin convertase [80] and belongs to the metallocarboxypeptidase family. Mice containing a (Ser202Pro) mutation in the CPE gene ($Cpe^{fat/fat}$) slowly develop obesity and mild type II diabetes [81]. High-throughput peptidomics analysis of $Cpe^{fat/fat}$ mice indeed revealed carboxyterminally extended intermediate peptides in addition to fully processed mature peptides, which are likely due to the activity of carboxypeptidase D [82–87].

Most bioactive neuropeptides are amidated at the carboxyterminus, which likely improves peptide stability and receptor recognition. This post-translational modification results from a multistep reaction that involves a hydroxylation of the glycine α-carbon by peptidyl-α-hydroxylating monooxygenase (PHM), followed by a cleavage reaction performed by peptidyl α-hydroxyglycine α-amidating lyase (PAL) to generate the α-amidated peptide and glyoxylate. In higher organisms, both enzymatic reactions are mediated by a bifunctional enzyme, peptidylglycine alpha-amidating monooxygenase (PAM) [88,89], while many invertebrates contain two distinct enzymes (PHM and PAL), that are encoded by different genes [90]. To our knowledge, no high-throughput peptidomics analyses have been reported on amidation mutants. However, a so-called "precursor and α-amidated peptide pairing" (PAPP) approach was recently described to reduce interpretation and computing time for database searching to identify α-amidated peptides from AtT-20 cells. Cells were cultured in the presence of a PAM inhibitor to enable the coexistence of fully processed α-amidated peptides and their glycine-extended precursor peptides, which differ 58 Da in mass but exhibit similar retention times and fragmentation patterns [91].

4.2. *Characterization of peptide processing enzymes in C. elegans by differential peptidomics*

Though the neuropeptide processing pathway has been characterized in many other organisms, little was known about the processing enzymes in *C. elegans*. Therefore, a simple though straightforward differential peptidomics approach was employed in the Schoofs lab to characterize key processing enzymes in *C. elegans* (Fig. 3). Briefly, peptides from *C. elegans* lines having a mutation in the presumed processing enzyme-encoding gene were monitored by LC–MALDI TOF MS as explained above. Though this strategy was not quantitative, it allowed easy comparison of peptide profiles to verify which genes are involved in the neuropeptide processing pathway.

In *C. elegans*, four genes were discovered that encode kex2-like proprotein convertases (KPC) by mining genome sequences and cDNA clones [2,92–96]. *C. elegans* KPC-2 is also named EGL-3 as mutants were initially discovered in a genetic screen for egg-laying deficiency about 30 years ago [97]. KPC-2 (or EGL-3) was initially suggested to be essential for neuropeptide processing by the Kaplan group [94]. When monitoring the peptide content of different *kpc-2* (or *egl-3*) mutants by mass spectrometry, very little ions corresponding in mass to neuropeptides could be observed, implying that the encoded enzyme is indeed required for the proper processing of bioactive peptides in *C. elegans* [49]. The involvement of *C. elegans* 7B2 (SBT-1, standing for *Seven-B-Two*) in the biosynthesis of at least some FLP and NLP peptides could also be demonstrated using a similar differential peptidomics approach [21].

Mutants for the *egl-21* gene were found in the genetic screen for egg laying deficiency that also yielded *egl-3* (or *kpc-2*) mutants [97]. Sequence similarity with carboxypeptidase E and a decreased immunoreactivity for the anti-RFamide antibody previously pointed to a role in the processing of FLP precursors [98]. 2D-nanoLC Q-TOF MS/MS analysis of *egl-21* mutants revealed 48 intermediate peptides with the basic amino acids still attached at the carboxyterminus, of which 37 NLPs and 11 FLPs; only 4 fully processed peptides could be detected [50]. When using the LC–MALDI-TOF MS approach, 90 carboxy-terminally extended peptides were identified in

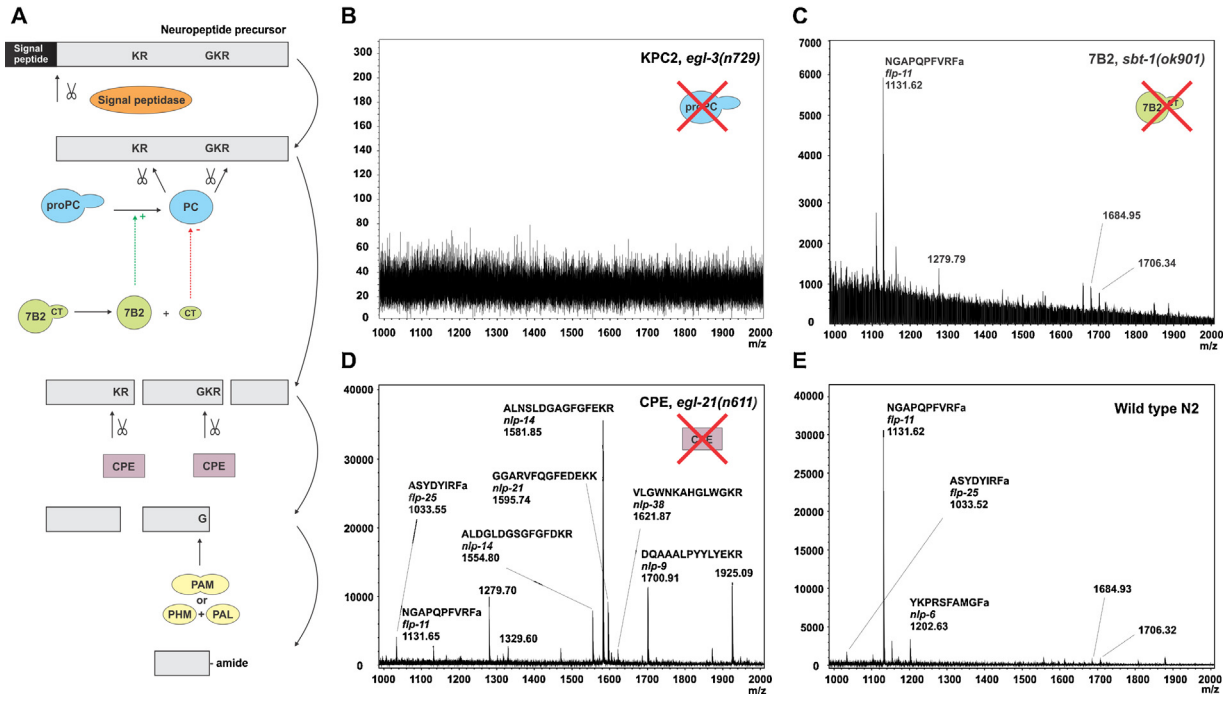

Fig. 3 – Characterization of neuropeptide processing enzymes in *C. elegans*. (A) Biosynthesis of (neuro)peptides requires extensive post-translational processing of an inactive peptide precursor protein. Comparison of MALDI-TOF MS spectra of corresponding LC fractions obtained from extracts from a proprotein convertase (KPC-2) mutant *egl-3(n729)* (B), a 7B2 mutant *sbt-1(ok901)* (C) and a carboxypeptidase E mutant *egl-21(n611)* (D) with wild type *C. elegans* (E) allowed characterization of these key processing enzymes [21,49,50].

addition to 24 fully processed peptides, which suggest the presence of an additional carboxypeptidase that acts redundantly or in parallel. We are currently characterizing mutants defective for presumed amidation enzymes.

5. Characterization of neuropeptide GPCRs in *C. elegans* and functionality

As indicated above, efforts of bioinformatics and biochemistry allowed identifying numerous peptidergic signalling molecules in the nematodes *C. elegans*, *C. briggsae* and *A. suum*, which is essential for identification and characterization of cognate G protein-coupled receptors (GPCRs) and further elucidation of signalling cascades that ultimately regulate diverse behaviours.

About 50 peptide receptors were initially mined from the *C. elegans* genome, based on sequence similarity with neuropeptide GPCRs from insects or vertebrates [1,2]. A number of them appeared to be involved in locomotion or reproduction, as determined by gene silencing using RNA interference [99]. However, 125 putative peptide GPCRs could be detected when using known peptide GPCRs as input sequences in a Multiple Expectation Maximization for Motif Elicitation/Motif Alignment and Search Tool (MEME/MAST) analysis [100]. This impressive list was recently refined by further updating the seeding set [4]. In order to find the cognate ligand of a receptor of interest, so-called reversed pharmacology approaches can be applied [101]. In short, the orphan GPCR is expressed in a heterologous expression system like Chinese hamster ovary (CHO) cells or human embryonic kidney (HEK) cells that also express a promiscuous G protein like the $G_{\alpha 16}$ subunit to yield a calcium flux that can be measured upon activation of the receptor. Cells are subsequently challenged with a library of synthetic peptides or fractionated peptide extractions. Calcium increases can be monitored by employing bioluminescent proteins like aequorin or by using fluorescent calcium indicators like Fluo-4. Alternatively, *Xenopus* oocytes can be used as cellular expression system in which gating of the co-expressed GIRK1 channel that interacts with free $G_{\beta\gamma}$ is measured by whole-cell voltage-clamp recordings upon receptor activation. Doing so, a total of 23 *C. elegans* GPCRs could be deorphanized (including splice variants). A thorough description of these GPCRs and their functionality in diverse physiological and behavioural pathways was recently reviewed [4].

6. Future directions: towards functional studies

Nematodes can exhibit a rich repertoire of different behavioural output responses upon diverse internal and external cues (including chemotaxis, thermotaxis, oxygen sensing and even associative learning). Understanding the neuronal substrates and mechanisms underlying signal input, processing and signal output, to ultimately direct proper behaviour, can be considered as an important goal in fundamental neuroscience. In recent years, distinct advances were made in the functional elucidation of defined peptidergic signalling systems in the model nematode *C. elegans* by

exploiting its advanced genetics toolkit in combination with different state-of-the-art techniques like Ca^{2+} imaging and optogenetics approaches.

Early experiments to probe the function of individual neurons mainly utilized the connectome diagram to select individual cells to be ablated with a focused laser beam in order to infer its role in a specific behavioural paradigm. Nowadays, optogenetics approaches allows researchers to reversibly activate and/or inhibit selected neurons in a time-resolved manner by simple illumination of the worm with blue, green or yellow light [102,103] while the activity of downstream neurons can be monitored by adopting Ca^{2+} imaging techniques [104–106]. In addition, automated tracking systems or video analysis tools are employed to monitor behavioural output [107]. However, it is important to note that most of these studies still rely on early knowledge from the mapped connectome or wiring diagram, while important neuronal communication is also achieved by means of slow-acting transmitters such as neuropeptides or biogenic amines that act beyond the neuronal synapses. Indeed, recent studies revealed that defined peptidergic signalling systems are required for fine-tuning or modulation of specific aspects of behaviour; see [4] for an extensive overview of all functionally described peptidergic signalling pathways in *C. elegans*. These recent advances in the functional elucidation of peptidergic modulation of neuronal circuits were possible by the ever-increasing technological platforms to probe the nervous system, but also relied on the availability of accurate peptide sequence information as revealed by peptidomics experiments.

Conflict of interest

None.

Transparency document

The Transparency document associated with this article can be found in the online version.

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